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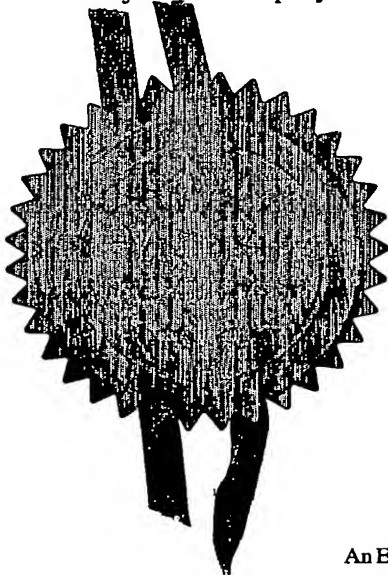
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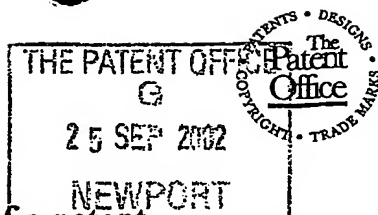
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1/77

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4. Title of the invention MUTANT RECOMBINASES

5. Name of your agent (if you have one) CRUIKSHANK & FAIRWEATHER
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Claim(s)

Abstract

Drawing(s) 5 + 5 *14*

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Mutant Recombinases

FIELD OF THE INVENTION

The present invention relates to a method of identifying hyperactive mutant recombinases and such mutant recombinases, as well as hybrid mutant recombinases. The present invention also relates to vectors comprising nucleic acid encoding said recombinases, as well as cells, especially eukaryotic cells capable of expressing said recombinases and carrying out site-specific recombination in the cell. Uses of said recombinases in biotechnology and/or gene therapy/transgenic applications is also provided, as well as novel recombination systems in a cell such as a eukaryotic cell, especially mammalian cell.

INTRODUCTION

Site-specific recombination is extensively used for genetic manipulations *in vivo*, and is central to many proposed approaches to gene therapy (Kilby *et al*, 1993; Nagy, 2000). It is generally used to site-specifically introduce or excise a DNA fragment (for example an engineered cassette) into or from the genomic DNA, in a controlled way (for example, at a specific stage of development, or following deliberate induction of the recombinase). Nearly all current applications of site-specific recombination in eukaryotes use the *loxP*-Cre system from bacteriophage P1 (see review by Nagy, 2000). Cre is a good recombinase for these purposes because of its short DNA recombination site (*loxP*; 34bp), its stability *in vivo* and the robustness of its activity even in chromatin-associated DNA. Use of these site-specific recombination systems in eukaryotes depends on the introduction of target DNA containing the appropriate DNA recognition/recombination sites into the organism. However, there is a great deal of current interest in modifying site-specific

recombinases so as to recognise natural sequences in eukaryote (e.g. human) genomes (Santoro and Schultz, 2002; Scimenti *et al.*, 2001; Buchholz and Stewart, 2001).

The bacterial transposon Tn3, a member of the large 'serine recombinase' family, encodes a site-specific recombination system comprising a 114 bp DNA site *res*, and a serine recombinase resolvase. *res* contains three binding sites for resolvase dimers. Recombination takes place within a 'synapse', consisting of the intertwined pair of resolvase-bound *res* sites that are to recombine. Strand exchange occurs at the centre of the two binding site Is, and is catalysed by the resolvase dimers bound at site I. However, wild-type resolvase is inactive on a substrate containing just two site Is; the presence of the 'accessory' resolvase-binding sites, II and III, hereinafter referred to as *acc* (Blake, 1995), in each *res* is essential for normal activity.

The *acc* sequences and the resolvase subunits bound to them play an essential part in the imposition of these selectivities (reviewed by Grindley, 2002). Regulatory DNA sequences like *acc* are prevalent in natural site-specific recombination systems. They may be adjacent to or distant from the site of crossing over, and may bind subunits of the recombinase (as *acc* does) and/or other proteins. Their functions are to ensure that recombination occurs only at the right times and places (reviewed by Nash, 1996).

The 20 kDA resolvases of the transposons Tn3 and $\gamma\delta$ are very similar (147 of 185 residues are identical). X-ray crystallography has yielded high resolution structures of $\gamma\delta$ resolvase, both on its own and in a complex with site I of *res* (Sanderson *et al.*, 1990; Rice and Steitz, 1994; Yang and Steitz, 1995;). However, the structure of the synapse is still not well-defined, despite much analysis. To build a functional synapse, at least three types of resolvase-resolvase interaction are thought to be required, two of which are represented in crystal structures. The 1,2 interaction

(Hughes *et al.*, 1990) forms the resolvase dimer that is present in solution and complexes of resolvase bound to parts of *res*; it is found in all of the three published crystal structures. The role of the 2,3' interaction between resolvase dimers, seen in crystals of the $\gamma\delta$ resolvase protein but not the DNA-resolvase co-crystal, is more elusive. Mutation of single residues at this interface eliminates recombination activity. The mutants are defective in cooperative binding to *res*, and in synapsis (Hughes *et al.*, 1990; Murley and Grindley, 1998). The 2,3' interaction is an essential feature of several proposed structures for the synapse (Rice and Steitz, 1994; Grindley, 1994; Yang and Steitz, 1995; Murley and Grindley, 1998; Sarkis *et al.*, 2001; Rowland *et al.*, 2002). A third interaction, not observed in any of the crystal structures, has been proposed to be required in order to bring two 1,2 dimers together in an arrangement suitable for catalysis of strand exchange at site I (Rice and Steitz, 1994; Yang and Steitz, 1995). This third interaction may also have other "non-catalytic" roles in synapsis. In the recent synapse model of Sarkis *et al.* (2001), the protein core comprises three "DNA-out" tetramers, interacting with each other at 2,3' surfaces.

In published work (Schwikardi and Droge, 2000), $\gamma\delta$ resolvase and a mutant of it have been shown to be active in mammalian cells, on full *res* sites and (very inefficiently) on the 28 bp site I of *res*. Another related recombinase, Gin, has been shown to be active in plant protoplasts (Maeser and Kahmann, 1991). Moreover earlier work by the present inventors describes mutants of Tn3 resolvase that act on 28 bp site I (inefficiently) in *E. coli* or *in vitro*. (Arnold *et al.*, 1999). Some more recent work has been disclosed in Sarkis *et al.* 2001). Nevertheless, there has been no disclosure of potential or actual use of these mutants in other organisms, e.g.

mammalian cells, or for genetic engineering purposes and moreover it is desirable to develop better mutant recombinases than those hitherto described.

While the concept of directing an enzyme to a chosen DNA sequence by attaching a DNA-binding domain from an unrelated protein may not be new; there are several examples of enzymes that have been fused to the Zif268 DNA-binding domain or derivatives, in order to direct activity to a new site, for example (Bibikova *et al.*, 2002, and references cited therein), this has not been done until now for any site-specific recombinase, because it is not obvious how to do it. However, sequence recognition by site-specific recombinases has been altered by mutagenesis, or by swapping domains between related proteins. The tyrosine recombinases Cre and FLP, for example, have been extensively mutated to try to achieve new sequence recognition, with partial success (Buchholz and Stewart, 2001; Santoro and Schultz, 2002; and references cited therein). Cre/Flp hybrid proteins with unusual properties (but no recombination activity) have been created (Shaikh and Sadowski, 2000), and phage lambda integrase has also been 'spliced' with a closely related protein in order to alter sequence recognition (Nunes-Duby *et al.*, 1994). However, for all the tyrosine recombinases, it is not obvious how the DNA-binding and catalytic functions of the protein could be separated, so changing recognition completely by attaching a heterologous DNA binding domain or similar is currently implausible.

For the serine recombinases, the crystal structure of $\gamma\delta$ resolvase bound to site I DNA (Yang and Steitz, 1995) shows that the 'DNA-binding' and 'catalytic' domains are folded separately and do not make an intimate interaction. It was previously known that the C-terminal 45 residues of Tn3/ $\gamma\delta$ resolvase (141-185) are largely responsible for specific DNA recognition, and that residues 1-140 contain the known catalytic functions. However, there is no suggestion in the art that catalysis

could be achieved without the natural C-terminal domain or some other similar domain. It was unlikely that specific catalytic residues were in the C-terminal domain, because several hybrid recombinases were active. In these hybrids, the C-terminal domain was exchanged for that of another quite closely related serine recombinase. The junction was so as to conserve exactly the positions of residues that were homologous in the two parents. Examples of hybrids were between parts of Tn3 and Tn21 resolvases, or Tn3 and Tn552 resolvases, or Tn3 and $\gamma\delta$ resolvases, or Gin and ISXc5 resolvase (Avila et al., 1990; Schneider *et al.*, 2000). Nevertheless, all of these hybrids were active only on long DNA sequences (full *res* sites), not on a short sequence like site I, and that only small changes in sequence recognition were achieved.

It is an object of the present invention to obviate and/or mitigate at least one of the aforementioned disadvantages.

It is another object of the present invention to provide novel mutant recombinases which may find use in gene therapy and/or other biotechnological applications and/or develop uses of mutant recombinases not hitherto suggested.

SUMMARY OF THE INVENTION

In a first aspect the present invention provides a method for identifying a hyperactive mutant serine recombinase capable of catalysing site-specific DNA recombination when bound to a recognition site comprising fewer nucleotides than necessary for achieving recombination with a corresponding wild-type serine recombinase, comprising the steps of

mutating said wild-type serine recombinase such that the mutant recombinase comprises one or more mutations, in a catalytic domain of the recombinase, with respect to the wild-type serine recombinase; and

detecting whether or not said mutant serine recombinase is capable of catalysing DNA recombination when bound to said recognition site comprising fewer nucleotides than necessary for achieving recombination with the corresponding wild-type serine recombinase.

The term hyperactive mutant recombinase is used to indicate that the mutant is capable of recombinase activity at smaller recognition sites than required by a wild-type recombinase.

Generally speaking recombination is carried out such that two such recognition sites are brought into close proximity for site-specific recombination to occur. Site-specific recombination is understood to relate to genetic recombination occurring between two particular, but not necessarily homologous, short DNA sequences, as in the integration or excision of phage DNA from a bacterial chromosome or in transposition. It is likely that more than one detection step from wild-type to preferred mutant may be required. That is it may be necessary to first select mutants with a substrate comprising one wild-type recognition site and one site of reduced size and to further mutagenise suitable mutants to get a preferred hyperactive mutant which shows recombination activity at two sites of reduced size.

Conveniently the sites of reduced size comprise less than 50 nucleotides, typically less than 30 nucleotides.

The present invention describes in one embodiment recombinases derived from Tn3 resolvase, by combining mutations as indicated below, that efficiently recombine two sequences corresponding to the 28 bp binding site I of Tn3 *res* (or

minor variants thereof). The D102Y E124Q mutant described in Arnold *et al.* 1999 has weak activity on a site I x site I substrate, in *E. coli* or *in vitro*; insufficient to be useful and is not therefore encompassed within the scope of the present invention. Much more active mutants were created by combining mutations in the region close to D102. Additionally, all the most efficient versions are mutant at D102. The present inventors have tested all possible residues at position 102; the effects of the single mutants are, in decreasing order of hyperactivity, Y, I > F, T, V, W > A > all others. Mutation of G101 has also been observed to cause a big effect; specifically to serine (G101S). Thus, mutants according to the present invention preferably comprise mutations at D102 and/or G101 or corresponding residues from other serine recombinases. Mutations at other residues can also promote hyperactivity; these include (in approximate order of strength of effect) V107M, V107L, Q105L, A117V, R121K, E124Q, E124A, A89T, F92S, M103I. Preferably the mutant enzymes have combinations of two or more of these mutations.

It has also been found that mutations of resolvase surface residues corresponding to a '2,3' interface' enhanced the activity of the mutants; see hereinafter. The mutations that have been tested were R2A and E56K, but mutation of several nearby residues (Hughes *et al.*, 1990) might be similarly effective. Thus, preferably the mutants of the present invention also comprise at least one mutation that effects the 2,3 interface.

Whilst the present inventors have focussed their work on Tn3 resolvase, it will be appreciated that the scope of the present invention may easily be extended to other serine recombinases, due to the similarity between members of the family.

The serine recombinases comprise a large family of related enzymes, which can be identified by sequence homology using standard algorithms such as BLAST.

Several residues are completely conserved, or nearly so, throughout the family. Structural features corresponding to particular parts of the primary sequence can be characterized because there are high-resolution crystal structures of the complete $\gamma\delta$ resolvase protein, and a fragment of Hin, as well as a large body of other biochemical data that give information on the structures. Those skilled in the art can easily identify the residues in other serine recombinases that might correspond to the Tn3 residues which can be mutated to cause hyperactivity. For example, residues G101 and D102 are the two Tn3 resolvase residues immediately preceding the N-terminus of a long α -helix, the E-helix of Yang and Steitz 1995, that contributes to the dimer interface. The equivalent residues can be identified in most other members of the serine recombinase family. Similarly, residues corresponding to those involved in the 2,3' interaction can be identified. See for example the review by Smith & Thorpe, 2002, or the attached alignment Figure 1 which shows an alignment of the N-terminal catalytic domains from a number of serine recombinases. For example, the present inventors have preliminary evidence that equivalent mutations of Sin recombinase from *Staphylococcus aureus*, which is quite distant from Tn3 resolvase, have the predicted effects.

The hyperactive mutants described herein can utilise the 'Site I' sequence for recombination. The 'Site I' is a 28 bp sequence from the natural *res* recombination site. Desirably smaller regions could be used which still cause recombination to occur. This may depend however on the mutant developed, but this can easily be determined by the skilled addressee. In practice, however, the sequence will always be embedded in a longer DNA molecule. It has been observed that many bases can be mutated individually without serious loss of recombination activity, and even multiple changes may not be very deleterious. However, a site comprising only the central 16 bp of site

I (that is, 6 bp at each end replaced so that no bases are conserved), or <16 bp, is not a substrate for the hyperactive mutant resolvases described herein.

Advantages of hyperactive serine recombinases over currently available enzymes for genetic manipulation

1. They act at short DNA sites, and do not require specific site orientation or supercoiling. They are therefore 'better' than other serine recombinases previously proposed for these uses. (Long sites and other requirements make it much more difficult to set up suitable constructs etc., and affect reactivity in chromatin-associated DNA).

2. They do not interact with tyrosine recombinases such as Cre or FLP, and act at different sites. So they can be used in applications where two (or more) independent recombination systems are required (see reviews etc.).

3. They may have advantages in real systems, because of their different properties and mechanism. For example, they might be more easily expressed/more stable in mammalian cells, or they might give more complete recombination.

In a further aspect the present invention provides a hybrid mutant recombinase comprising an N-terminal catalytic domain from a serine recombinase connected by way of a linker region to a heterologous C-terminal DNA binding domain wherein the mutant recombinase is capable of binding nucleic acid by way of said DNA binding domain and said mutant recombinase catalysing recombination.

Preferably the catalytic domain is from a hyperactive mutant recombinase identified, for example, according to the present invention.

It was previously known that the N-terminal domain of Tn3 resolvase (or any other serine recombinase tested) has no catalytic activity on its own, nor does the

isolated N-terminal domain of mutants that act on site I. It was therefore surprising that attachment of an unrelated DNA-binding domain to a mutant catalytic domain could restore activity at a very different DNA site. Reasons why this might not have been considered feasible are:

1. The natural DNA-binding domain might play some essential part in the reaction mechanism, which could be performed by a related DNA-binding domain, but not an unrelated one; e.g. involvement of conserved residues, or transient dissociation from its binding site.

2. The natural domain might not participate in the reaction, but its size, shape, and position might be critical. For example, a larger domain might interfere with essential conformational changes in the DNA or protein.

3. The nature of the linker sequence between the two domains might be critical, and it might not have been possible to reconstruct it appropriately (for example, because the N-terminal residues of the unrelated DNA-binding domain and the resolvase DNA-binding domain were differently positioned relative to the binding site).

In practise the important steps in going from a natural serine recombinase eg. Tn3 recombination system to a functional 'hybrid' system are as follows:

1. Identification of multiple mutants of resolvase that rapidly recombine two 28 bp site I's, thereby removing the requirement for 'accessory sites' (see Arnold *et al.*, 1999; and the development of hyperactive mutants described herein);

2. Deciding where to terminate the N-terminal domain, to separate DNA-binding from essential catalytic functions;

3. Choosing of an appropriate substitute DNA-binding domain (e.g. Zif268) (from literature analysis);

4. Designing appropriate linker peptide sequences, to join the DNA-binding and catalytic domains of the hybrids; and

5. Designing of potential recombination sites for the hybrid enzyme.

In the hybrid recombinases of the present invention, the catalytic domain of a hyperactive mutant resolvase (or other serine recombinase) is joined via a short linker sequence to a DNA-binding domain from a different protein. The DNA-binding domain can be any of a number of such domains known to those skilled in the art, such as the domain from other serine recombinases, or from some transposases, or from bacterial repressors, tyrosine recombinases, etc. Suitably the DNA-binding domain may be eukaryotic in origin, for example, from eukaryotic transcription factors, especially a zinc finger DNA-binding domain such as that from Zif268, or variants of one of these with altered sequence recognition.

The hybrids that have been constructed to date, by the present inventors contain the contiguous first 146 residues of Tn3 resolvase, with appropriate 'activating' mutations see hereinabove for information. (The proteins actually tested have all of the following mutations: R2A E56K G101S D102Y M103I Q105L although this should not be construed as limiting). The traditional 'catalytic' and DNA-binding' domains of resolvase and relatives were identified following proteolysis, and are residues 1-140 and 141-183 (for $\gamma\delta$ resolvase) respectively. The C-terminal domain has been shown to retain DNA-binding activity, but no activities were found for the N-terminal 'catalytic' domain on its own. Current evidence suggests that all catalytic functions may reside in the contiguous residues 1-125. The sequence from 126-146 may however, contribute to binding and sequence recognition near the centre of the site. It is envisaged that it may be possible to mutate the 126-

146 region or replace it with the equivalent segment from another serine recombinase, to alter reactivity or target specificity.

Preferably the linker region should be a sequence with structural flexibility, but the linker may depend strongly on the DNA-binding domain employed. This can however, easily be determined by the skilled addressee. It may be that shorter linkers will potentially lead to more efficient recombination, but might be more restricted in sequence variation. Thus an appropriate linker may depend on the requirements of the user. Some linkers may increase the efficiency of recombination at the expense of DNA sequence specificity, whilst others may allow recombination to occur at lower efficiency, but with a greater variation in sequence.

Resolvase binds to site I as a dimer. To act at asymmetric sequences (see below), it will be desirable to bind a heterodimer of the hybrid recombinase, where the DNA-binding domains of the two subunits interact with distinct sequence elements. Likewise, it may be desirable to have a different heterodimer to recognize a partner recombination site; i.e. up to four different hybrid recombinase proteins could be simultaneously involved, see Bibikova *et al.*, 2002 for an example of this type of approach in a different system).

Although the hybrids of the present invention have been exemplified with respect to Tn3 resolvase-derived systems, this should not be construed as limiting. Based on the present teaching similar procedures could be used to create equivalent hybrids from other serine recombinases. Indeed, this might lead to better recombinases, because other recombinases have different 'site I' central sequences, which could be better for some specific natural sequences chosen to be recombination sites.

In order for a hybrid recombinase to function appropriately and catalyse recombination it is necessary for the enzyme to recognise and bind to an appropriate stretch of DNA. Typically the DNA sequence may comprise two regions recognized by the DNA-binding domain(s) of the hybrid recombinase(s), flanking a central sequence which may make some specific interactions with the catalytic domain and/or the 126-146 segment, or similar region from another serine recombinase. The site will always be embedded in a longer DNA molecule (typically, but not necessarily, kilobasepairs).

A typical site may be about 40 bp long. Experiments by the present inventors indicate that the positioning of the sequence elements that recognize the DNA-binding domains (relative to the centre of the site) is very important. The ideal positions will certainly vary depending on the DNA-binding domain and linker sequence used. The sequences of sites that have been tested by the present inventors are shown in attached Figure 2a. These sites all comprise two copies of the natural 9 bp motif that is recognized by Zif268, flanking a central sequence of varying length. All of the central sequences used so far contain at least 11 contiguous basepairs of identity to the centre of site I, but it is very likely that sequences with less similarity to site I will also be active. It should be noted that non-hybrid hyperactive mutant resolvases are not active on these sites. The main features of the recombination site are illustrated in the attached Figure 3.

The two sites that are to recombine need not be identical. They could be recognized by separate hybrid recombinase heterodimers, providing that the catalytic domains were similar, so that the catalytically competent synapse of the two sites could be formed. Importantly however, the 2 bp at the centre of the sites should be identical for efficient reaction (this is because these bp form a 'heteroduplex' in the

recombinants, and the basepairs would be mismatched if the 2 bp sequences were different). (Tyrosine recombinases require longer regions of identity at the centre of their sites; 6 bp for Cre, and 8 bp for FLP). Also, the relative orientation of this 'overlap' sequence defines whether excision or inversion will occur between two sites in the same molecule.

Without being bound by theory it is predicted that, for any chosen recombination site sequence, it will be necessary to carry out an optimization procedure to achieve high activity. In general, this procedure will include the following steps.

1. One or two candidate recombination sites will be chosen, which have a central sequence with some similarity to site I, flanked by sequences at appropriate distances from the centre that could recognize selected DNA-binding domains;

2. The DNA-binding domains will be optimized for recognition of their targets. This can be done completely separately from the recombination system, using methods well known to those skilled in the art; mutagenesis followed by 'phage display' selection, swapping of parts from known variants of the DNA-binding domain, etc. (see reviews; e.g. Pabo *et al.*, 2001);

3. Likewise, the catalytic domains and linkers may be optimized for interaction with and recombination at the central sequences. This may be done by making a trial recombination site, with the chosen central sequence placed between motifs recognized by a DNA-binding domain that is known to work well; for example, Zif268 itself. The catalytic domain and linker will then be optimized in essentially the same way as in (2), using mutagenesis/selection methods (e.g. as described in herein.), or splicing of parts from different variants or from different serine recombinases, etc; and

4. Complete candidate hybrid recombinases may then be assembled, and tested on the intact chosen sites. If necessary, efficiency of recombination at the sites may be improved by further rounds of mutagenesis and selection.

In a further aspect there is provided use of a hyperactive mutant recombinase, or hybrid recombinase according to the present invention for carrying out site-specific recombination.

Preferably site-specific recombination is carried out in a eukaryotic cell or on eukaryotic DNA. More preferably site-specific recombination is conducted in a mammalian cell or on mammalian DNA.

In a further aspect there is provided use of a hyperactive mutant recombinase, or hybrid recombinase according to the present invention for the manufacture of a medicament for therapy or prophylaxis. Said hyperactive mutant may be used to introduce a therapeutic gene or replace/remove a defective or deleterious gene sequence from the genome of a particular organism, such as a mammal.

In principle, all of the recombinases described herein could be used for virtually any current or envisaged applications of site-specific recombinases such as cell therapy, tissue engineering and/or gene therapy (see for example Gorman & Bullock, 2000 and references cited therein). The hybrid recombinases can also be used to create new sequence specificities in experimental systems, but more importantly, they can be used to target recombination to natural sequences in the genomes of (any) important organisms.

Advantage and utility of hybrid recombinases

Potential applications are for example in WO 01/16345. Basically, a DNA segment containing useful (e.g. therapeutic) genes can be introduced at specific

genomic sites, or 'bad' genes can be excised from the genomes of living cells, or control of gene function can be systematically altered by excision, integration, or inversion of DNA segments. Two examples are: (1) It may be possible to develop a potential therapy for HIV and other retroviral diseases, by excision of the proviral DNA (see below); (2) it may be possible to introduce useful genes (e.g. for antibodies) at specific sites in for example the casein gene loci of cows, so that they would express large quantities of the gene product in their milk.

Several groups are attempting to adapt the tyrosine recombinases Cre and FLP to recognize new sites (see, for example, Buchholz and Stewart, 2001; Santoro and Schultz, 2002). However, the present inventors believe that mutant serine recombinases are likely to be much more successful for this approach, because their modular structure facilitates the 'hybrid' constructions described herein. Also, they are likely to be much more suitable for recombining between two natural sites (e.g. for excision of natural genes), because they require only 2 bp of homology at the centre of the recombination sites for efficient reaction. Cre requires 6 bp and FLP requires 8 bp (Nash, 1996); pairs of sites with this degree of identity will be very rare. Clearly the recombinase genes would need to be introduced into and expressed in the target cells, for most applications. Thus the present invention also provides a vector comprising a nucleic acid encoding a hyperactive or hybrid mutant recombinase according to the present invention. The vector may also comprise one or more recombinase binding sites which are recognisable by said mutant recombinase. Said recognition site(s) may comprise a mutated sequence with respect to the native sequence recognisable by the unmutated recombinase. The present invention also provides a cell which has been transformed with a vector as described above. It might be necessary to modify the recombinases for various reasons concerned with their

properties in the target cells, such as to increase stability, direct them to the nucleus, allow their visualization. All such modifications are well known to those skilled in the art.

The present invention will not be further described by way of example and reference to the Figures which show:

Figure 1 shows a sequence alignment of the N-terminal domain of a number of serine recombinases, showing that they are quite conserved;

Figure 2a shows details of Z-box sites which have been tested by the present inventors;

Figure 2b shows details of the flexible linkers which have been tested by the present inventors;

Figure 3 shows a schematic representation of a generic hybrid recombination site;

Figure 4 shows plasmids used for *in vivo* screening for mutants. The *repA* gene product is required for initiation of replication at the pSC101 origin. See Arnold *et al.* (1999) for further details. Pgal(*res* x *res*) is shown. In pGal(*res* x I), *res* A has been replaced by a fragment containing site I, and in pGal(I x I), both *res* sites have been replaced by site I fragments. PStr(I x I) is similar to pGal(I x I), but the sequences containing the *galk* gene are replaced by sequences conferring resistance to tetracycline and sensitivity to streptomycin (see Materials and Methods section);

Figure 4b shows *in vivo* properties of resolvase mutants. The colour of colonies on MacConkey agar plates is shown. Red signifies no resolution activity, pale yellow signifies full resolution, and pink signifies slow resolution. Some mutants gave mixtures of colonies of different colours (shown as a sector circle). Detection

of weak activity of some mutants on certain substrates was variable, depending on factors such as colony density (red circles marked with a + sign);

Figure 5 shows (Structures of pC2*res*, pC*res*I, and pC2I = pMA21, pAL265, pAL225)

a) Summary of *in vitro* properties of some multiple mutants of resolvase. -=no activity. Higher activity is indicated by more + signs. The *in vitro* activities of D102Y, E124Q, and D102Y E124Q mutants are described in Arnold *et al.* (1999);

Figure 6 shows the location of the mutants which have been carried out by the present inventors; and

Figure 6b shows residues 100-125 of resolvase subunit A (Yang and Steitz, 1995), containing the N-terminal section of the E-helix and the immediately preceding residues, are shown in backbone representation (green). The view is from the same angle as in Figure 2a. The sidechain of D102 is shown in blue, and the sidechains of other residues mutated in the hyperactive proteins are in red. Interactions of these residues are denoted by the thick lines. Residues in the same subunit are green, and those in subunit B are in orange.

MATERIALS AND METHODS

Mutagenesis

Designed mutations were introduced by cloning appropriate double-stranded synthetic oligonucleotides into pAT5 (Arnold *et al.*, 1999), or pMA5811, which was derived from pAT5 by deletion of an *EcoRV*-*NruI* fragment. Random mutations were created using synthetic oligonucleotides as described in Arnold *et al.* (1999), or by the polymerase chain reaction. Primers flanking the complete resolvase ORF of pAT5 or pMA5811 were used to amplify the fragment, and mutagenesis was caused by biasing the proportions of the dNTPs (Fromant *et al.*, 1995), or by introduction of 8-oxodGTP

or dPTP nucleotides (Zaccolo *et al.*, 1996). Appropriate restriction digest fragments from mutagenized DNA were cloned into pAT5 or pMA5811 to create libraries of mutants which were screened as described below.

Screening and selection

Resolvase expression plasmids, *in vivo* expression, and the GalK-based screening method were as described in Arnold *et al.*, (1999). pGal(*res* × *res*), pGal(*res* × I), and pGal(I × I) were described by Arnold *et al.* as pDB34, pDB37, and pDB35 respectively. Typically, between 1 000 and 10 000 candidate mutants were screened. The numbers were limited either by the diversity of the library, or by the screening procedure, in which 'white' colonies could not be picked reliably when there were more than ~1 000 colonies on a single 8 cm diameter MacConkey agar plate. Some mutants were selected by a method in which resolution of a test plasmid causes loss of tetracycline resistance, but confers resistance to streptomycin. In the test plasmid pStr(I × I) (=pMA5531), the *galK* gene of pGal(I × I) was replaced by sequences containing a gene for tetracycline resistance, and the *strA* (*rpsL*) gene, encoding the wild-type ribosomal S12 protein from pABS12. When highly expressed, S12 causes streptomycin-sensitivity in strains of *E. coli* that are normally resistant due to a mutation in the chromosomal copy of this gene. Agar plates containing streptomycin and kanamycin therefore select for loss of the plasmid-encoded *strA* gene by recombination between the two site Is. Libraries of pAT5 containing mutant resolvase ORFs were used to transform *E. coli* strain DS941/pStr(I × I). Liquid cultures (LB medium) were grown without selection for variable time intervals before spreading aliquots on L-agar plates containing kanamycin and streptomycin (200 µg ml⁻¹). Mutant versions of pAT5 were isolated from colonies that appeared at early time points.

RESULTS

Mutation strategies

The point mutation D102Y allows Tn3 resolvase to recombine a *res* × site I substrate. The double mutant D102Y E124Q can slowly recombine a site I × site I substrate, although it is still greatly stimulated by the presence of *acc* (in a *res* × *res* or *res* × site I substrate) (Arnold *et al.*, 1999). The present inventors therefore adopted three approaches to find other activating mutations of resolvase: (A) random mutagenesis of the catalytic domain of resolvase; (B) mutation of residue D102 to all other amino acids; (C) random mutagenesis of resolvases which already contained D102Y, E124Q, or both mutations. The inventors then observed the effects of combining activating mutations with each other and/or with mutations at the 2,3' interface.

Random mutagenesis of the resolvase catalytic domain

Libraries of resolvase expression plasmids mutagenized throughout the catalytic domain (residues 1-140) by PCR-based methods (see Materials and Methods), or between residues 94 and 121 with oligonucleotides (Arnold *et al.*, 1999), were screened for resolution of pGal(*res* × I) *in vivo*, by an assay in which resolution of a test plasmid, with a gene for GalK flanked by two recombination sites, is detected by formation of white (*galK*⁻) rather than red (*galK*⁺) colonies on MacConkey indicator agar plates (Arnold *et al.*, 1999; Figure 4a). Sequencing of the resolvase expression plasmids from white colonies identified several hyperactive mutants, all of which were altered at residue 102 (Table 1). The present inventors noted that the single mutant M103I was erroneously stated to be hyperactive in Arnold *et al.* (1999); re-sequencing of the expression plasmid revealed an additional mutation D102A. M103I does not show detectable hyperactivity in the MacConkey assay, nor does it when combined with E124Q (see above; Figure 4b). However, the D102A M103I and D102T M103T double mutants are more hyperactive than the corresponding D102 single mutants (see below).

The G101S and Q105L single mutants were later found to be hyperactive (Figure 4b; see below), though they were not recovered from screens of mutagenized wild-type resolvase, probably because their resolution of pGal(*res* × I) was insufficient to produce distinctly paler single colonies amidst many red colonies.

Saturation mutation of D102

Residue D102 was mutated to all 19 other amino acid residues, by cloning synthetic oligonucleotides into the resolvase ORF of pAT5. The mutants were assayed as described above; the results are summarized in Figure 4b. All 19 mutants resolved pGal(*res* × *res*) which has two full *res* sites. pGal(I × I), a plasmid with no *acc*, i.e. just two copies of site I, was not resolved detectably in this assay by any D102 mutant. pGal(*res* × I) was resolved efficiently by the mutants D102Y, D102F, and D102I. D102W, D102V, and D102T had lower activity on pGal(*res* × I), as indicated by a pinker colour of the colonies in the assay, D102A had barely detectable activity, and all other D102 mutants did not have detectable activity (i.e. red colonies). The mutant D100Y was also tested, but was not hyperactive (see Discussion).

To assess further the effects of the activating D102 substitutions, some of them were combined with E124Q, and the properties of the double mutants were compared (Figure 4b). These results suggest that the most potent activating mutations of D102 are to F, Y, or I.

Random mutation of D102Y, E124Q, and D102Y E124Q ORFs

The entire D102Y resolvase ORF was subjected to random mutagenesis by PCR-based methods. Mutants which resolved pGal(I × I) retained D102Y, and had the additional mutations A117V or R121K or E124Q (Table 1; Figure 4b). The original A117V isolates had a third mutation, I138V. The D102Y A117V double mutant was active on pGal(I × I), but less so than the original triple mutant. I138V was not hyperactive as a single mutant, and the D102Y I138V double mutant did not resolve pGal(I × I) (data not shown). The D102Y E124Q double mutant had been created

previously by design (Arnold *et al.*, 1999). The single mutants A117V, R121K, and E124Q resolved pGal(*res* × *res*), but resolution of pGal(*res* × I) or pGal(I × I) was undetectable.

E124Q resolvase was mutagenized by PCR, between residues 10 and 140. Libraries of mutants were screened for resolution of pGal(*res* × I), which is not resolved by E124Q itself. Second mutations which confer resolution activity were identified as F92S, G101S, D102V, D102Y, and Q105L (Table 1; Figure ***b). The G101S, D102Y, and Q105L (+E124Q) double mutants also resolved pGal(I × I). All of the derived single mutants except F92S had detectable activity on pGal(*res* × I) (Figure 4b). Some other D102 mutants had increased hyperactivity when combined with E124Q (see above).

The entire resolvase ORF containing both D102Y and E124Q mutations was mutagenized by PCR. Because D102Y E124Q itself resolves pGal(I × I), an alternative method was used to select for *E. coli* containing resolvase mutants that were able to promote rapid resolution of a site I × site I plasmid pStr(I × I), thereby conferring streptomycin resistance (see Materials and Methods for details). Three mutants which rapidly resolved pStr(I × I) (and pGal(I × I)) had the additional mutations A89T, G101S, and V107M (Table 1). The derived double mutants A89T D102Y, G101S D102Y, and D102Y V107M all resolved pGal(I × I) (A89T D102Y less efficiently - pink colonies). A89T and V107M single mutants did not show detectable hyperactivity in the MacConkey plate assay (Figure 4b).

Combinations of activating mutations

The results described above indicated that some combinations of activating mutations were more effective than single mutations. The present inventors therefore tested resolvases with several designed combinations of mutations, by making 'cassettes' containing either four mutations of residues at or near D102 (G101S D102Y M103I Q105L; M-cassette), or three mutations nearer the C-terminus (A117V R121K E124Q; C-cassette). Further mutants were then created by combining the

cassettes with D102Y, E124Q, or each other (Figure 4b). The M-cassette mutant promoted efficient resolution of all three pGal test plasmids. Combination of the M-cassette with E124Q (MQ) decreased activity on pGal(*res* × I) and pGal(I × I). The C-cassette mutant did not promote detectable resolution of any of the pGal plasmids, nor did the mutant containing both M- and C-cassettes (MC). Combination of the C-cassette with D102Y (YC) restored resolution of pGal(*res* × *res*), but the other pGal plasmids were not resolved.

Effect of mutations at the 2,3' interface

Mutations of residues at the 2,3' interface abolish recombination activity in $\gamma\delta$ resolvase. Tn3 resolvase mutant in two 2,3' interface residues (R2A and E56K; N-cassette; see Hughes *et al.*, 1990) was likewise completely inactive, *in vivo* (Figure 4b) and *in vitro* (unpublished results). The triple mutants R2A E56K D102Y (NY) and R2A E56K E124Q (NQ) were also inactive, but in striking contrast, the quadruple mutant R2A E56K D102Y E124Q (NYQ) was hyperactive; more so than the double mutant D102Y E124Q. The M and MQ multiple mutants (see above) were also combined with R2A E56K (N-cassette), creating NM and NMQ multiple mutants. These proteins efficiently resolved all three pGal test plasmids.

In vitro properties of multiple hyperactive mutants

Several of the hyperactive resolvases were over-expressed and purified. Their *in vitro* activities are summarized in Figure 5, and broadly agree with the phenotypes observed *in vivo* (Figure 4b). The present inventors analysed the multiple mutant M resolvase and its 2,3'-defective derivative NM in detail. Both resolvases were active on a site I × site I supercoiled plasmid pTet(I × I) *in vitro*, but NM resolvase was significantly more active. About half of the substrate was recombined in 4 minutes, a rate similar to that of wild-type resolvase on the standard resolution substrate pTet(*res* × *res*) under similar conditions. Site I of *res* is functionally symmetric (Bednarz *et al.*, 1990), so as expected NM resolvase gave about equal amounts of resolution and

inversion products from pTet(I × I). There was no evidence of topological selectivity; a series of knots and catenanes, consistent with random collisions of sites, was formed from single pTet(I × I) molecules, as well as products of recombination between sites on separate molecules. Unexpectedly, *acc* sequences inhibited recombination by N and NM resolvases; recombination of pTet(*res* × I) was slow, and recombination of pTet(*res* × *res*) was even slower. These mutants do not use *acc* to impose selectivity. Resolution was not preferred over inversion, and the distributions of product topologies from all three pTet plasmids were similar; there was no evidence of a preferred 2-noded catenane product from pTet(*res* × I) or pTet(*res* × *res*). M and NM resolvases also promoted rapid intra- and intermolecular recombination between site Is on linear DNA molecules (unpublished results).

DISCUSSION

The role of *acc*

Wild-type resolvase binds to a substrate containing two copies of site I, but does not catalyze any recombination. *Acc* sequences correctly positioned adjacent to both site Is (that is, a *res* × *res* substrate) are essential for efficient catalytic activity (Bednarz *et al.*, 1990). Hyperactive mutants promote recombination between two site Is in the absence of one or both of the *acc* sequences. The mutations very likely disrupt, or allow circumvention of, a natural regulatory mechanism which enforces *acc*-dependence, rather than conferring an intrinsically new functionality. Possible ways in which they might do this are considered in the next section.

It was expected that hyperactive resolvase-mediated site I × site I recombination in plasmids would be topologically non-selective (see Arnold *et al.*, 1999), because topological selectivity of wild-type resolvase involves its interactions with *acc* (see Introduction). More surprisingly, *in vitro* recombination by M, NM, and some other hyperactive mutant resolvases was inhibited by the presence of *acc* sequences in *res* × *res* or *res* × site I substrates, and the mutants do not use *acc* to specify a single product topology. Without wishing to be bound by theory, the present

inventors speculate that subunits of these mutants bound at *res* tend to make inappropriate interactions with each other or with subunits bound at a partner recombination site, which inhibit recombination and disable the proper function of *acc*.

Activating mutations

The present screens have been sufficiently thorough that the inventors are confident that all or nearly all the residues that can be mutated to give hyperactivity have been identified. They are all in the catalytic domain of resolvase, between amino acid residues 89 and 124 (except for the weakly enhancing mutation I138V) (see Figure 6). This region comprises the last two strands of the β -sheet that forms the core of the catalytic domain, the N-terminal part of the E-helix, and short connecting loops. Many of the residues in this segment of the polypeptide sequence are involved in the interface between the two subunits of the resolvase 1,2-dimer.

D102 is the only single residue mutant which can promote complete resolution of pGal(*res* \times I). The long E-helix which is a major component of the dimer interface begins at residue 103, and residues 99-102 make a loop connecting the E-helix to the C-terminal strand of the catalytic domain core β -sheet (Yang and Steitz, 1995). D102 (E102 in $\gamma\delta$ resolvase) does not contribute to any of the known resolvase interfaces (see Arnold *et al.*, 1999, for more details). Mutating D102 to all other amino acids, remarkably, all 19 mutants resolved pGal(*res* \times *res*). Seven mutants were observed to be hyperactive. The sidechains of the activating substitutions (Y, I, F, V, T, W, and A, in approximate order of decreasing effect) are all uncharged and hydrophobic, but other hydrophobic residues (M, L, and P) do not activate detectably. The mutation G101S, of the residue preceding D102, is also activating, and the double mutant G101S D102Y promotes complete resolution of pGal(I \times I), with no *acc*.

Only one other single mutant, Q105L, was detectably hyperactive in the MacConkey assay. The equivalent $\gamma\delta$ resolvase residue, K105, is within the E-helix, but apparently does not participate in the dimer interface. The nearby activating

mutation V107M maps to $\gamma\delta$ resolvase residue V107, whose sidechain is in a very different environment - deeply buried in the hydrophobic centre of the dimer. It interacts with residues in the N-terminal 'subdomain' (residues 1-100; see below) of its own subunit, and with the E-helix of the other subunit of the dimer.

The sidechains of the three residues A117, R121, and E124 are on the same face of the E-helix and contact the partner subunit of the dimer, at or near its presumptive catalytic site (Yang and Steitz, 1995;). This group of interactions is present only once in the crystal structure of the DNA-bound 1,2 dimer, being one of its most obvious asymmetric features. The mutations A117V, R121K, and E124Q are all conservative changes, whose activating effect (in Tn3 resolvase) is only manifested in the presence of at least one other activating mutation (D102Y in all cases tested).

Three other mutations, A89T, F92S, and I138V, had an enhancing effect when combined with other activating mutations (Figure 4b). A89 (S89 in $\gamma\delta$ resolvase) is on the surface of the resolvase dimer. It has been noted to be on the putative interface of dimers in the 'DNA-out' tetramer (Sarkis *et al.*, 2001). The F92 sidechain makes various hydrophobic interactions, including one with the E-helix (L111) of its own subunit. Residue I138 (V138 in $\gamma\delta$ resolvase) is the second residue beyond the C-terminal end of the E-helix; its sidechain contacts a deoxyribose of the DNA backbone in the minor groove. Possibly the enhancing effect of the I138V mutation is due to suppression of an undesirable interaction, as suggested for mutations at the 2,3' interface (see below).

The identification of multiple mutants with stronger hyperactivity led the present inventors to construct two 'cassettes' with groups of mutations which were close to each other in the primary sequence. Resolvases with all four mutations G101S, D102Y, M103I, and Q105L promoted rapid recombination of site I \times site I plasmids, *in vivo* and *in vitro*. In contrast, catalytic activity of resolvases with the three mutations A117V, R121K, and E124Q was severely reduced.

The *acc*-independent activity of hyperactive mutants is stimulated by additional mutations at the 2,3' interface. The 2,3' interface is clearly therefore not required for the catalytic steps of recombination (see also Grindley, 1993; Murley and Grindley, 1998; Sarkis *et al.*, 2001). A recent model for the synaptic complex (Sarkis *et al.*, 2001) proposes that resolvase dimers bound at site I make 2,3' interactions with subunits in the rest of the synapse. Again without wishing to be bound by theory, the present inventors speculate that this interaction is pivotal to the mechanism of activation of catalysis in the natural system, but it may be superfluous and mildly inhibitory for mutants that do not require *acc*.

Development of hybrid recombinases

Hybrid recombinases have been developed which comprise a Tn3 resolvase catalytic domain linked to a zinc-binding domain, Zif268. All the recombinases tested comprise residues 1-144 of the resolvase mutant "RMMD+", which has the following changes from wild-type; R2A, E56K, G101S, D102Y, M103I, Q105L. The first two mutations are to the "2,3'" interface, and the other 4 are "activating" mutations. In all cases the Zif268 domain has the wild-type sequence starting from residue 2 as given in the crystal structure paper (N.P. Pavletich and C.O. Pabo, Science 252, 809 – 817 (1991). Between residue 144 of resolvase and residue 2 of Zif268 there is a "linker" sequence. The sequences tested are shown in Figure 2b. All proteins show activity; the most active in *E. coli* was the one with the linker marked with a big asterisk.

The sites used are also shown in Figure 2a. The relevant ones are those marked Z0, Z+2, etc. They comprise two invariant 9 bp motifs recognized by Zif268 (pale blue boxes with three little arrows inside), flanking a central invariant sequences made up of at least 13 bp of sequence from the centre of res site I (darker pink shading), and some varied "spacer" basepairs which change the distance between the

two Zif268-binding motifs. The site marked with the big asterisk (Z+6) gave the highest recombination in *E. coli* (about 75% of substrate recombined after about 20 generations of growth). Z+4, and Z+8, 10, 12 also showed activity. The Z0 and Z+2 sites were inactive.

All combinations of hybrid proteins and sites shown in Figures 2a and b have been tested in *E. coli*. The Z0 and Z+6 sites, and two hybrid proteins corresponding to the linker marked with the asterisk and the one at the top of the list, have been tested in vitro. In vitro, the Z+6 site recombines much better than the Z0 site, which is almost inactive.

Based on the detailed knowledge of Tn3 site-specific recombination and mutants described above, the intention is to design novel systems for promotion of DNA rearrangements in for example higher eukaryote cells. It is first necessary to test and show that resolvase mutants can recombine substrates containing two copies of a minimal 28 bp recombination site ('site I'), in mammalian cell lines. The methods to be used are quite well established, Groth, et al. 2000 and Schwikardi and Dröge, 2000. Experiments may initially be in two or three standard cell lines, for example COS-1, 3T3, or 293 cells. A mutant resolvase will be expressed in the mammalian cells from a suitable plasmid derived from available vectors, with a standard promoter such as the SV40 early or CMV immediate early viral promoters, and a transcription terminator/polyadenylation signal. Further experiments may involve quantitative estimation of the extent of recombination, using constructs in which recombination changes expression of a reporter gene (eg. luciferase and/or GFP). To determine the cellular localization of the mutant resolvases, it is possible to create fusions with green fluorescent protein (GFP) by established methods, and analyse cells expressing the fusion proteins by microscopy. These fusion proteins will also allow easy

determination of transfection efficiency. The present inventors have already demonstrated full recombination activity by resolvase-GFP fusion proteins *in vitro* and in *E. coli*. If it proves to be desirable, it is possible to attach a nuclear localization signal to the resolvase coding sequence.

It is then possible to compare the efficiencies of existing hyperactive resolvase mutants, to identify the features of the recombinase that are most important for efficient recombination at minimal sites in the cell lines, and to create potentially improved versions of the system. Further optimization of recombination activity may be achieved by selection strategies, which will be easily adapted from established methods for selection of resolvase mutants in *E. coli* see above. For example, it is possible to make a construct that contains a gene for a hyperactive resolvase, adjacent to a pair of recombination sites flanking a marker gene. Libraries of mutants in the resolvase ORF may be created, e.g. by PCR mutagenesis and *in vitro* 'shuffling'. Cassettes which recombine upon transfection into mammalian cells can be recovered by PCR amplification, and are likely to encode an active resolvase. The sequences encoding the active resolvases can be subjected to further mutagenesis if required. The same plasmid constructs can be used for selection experiments in *E. coli*.

The natural *res* site I is functionally symmetrical, so either excision or inversion can occur in a substrate with two sites. Alteration of the 2 bp sequence at the centre of site I can break this symmetry, so that only one type of event (resolution or inversion) is allowed; this restriction might be desirable for most biotechnology applications, and it is straight-forward to test the properties of such sites in cell lines. It is predicted that other simple mutations in the sequence of site I might increase efficiency of recombination in the cell lines, and reduce the likelihood of reversal of the rearrangement by a second round of recombination.

Successful demonstration of efficient recombination following co-transfection may be followed by more stringent tests requiring excision of a marker gene which is inserted into the chromosomal DNA at random sites. This will more accurately reflect the situation in applications of the system, when the sequences of interest are typically associated with nuclear chromatin. Random integrants could be created by transfection of a suitable plasmid substrate followed by selection for a gene encoded by the plasmid, for example neomycin resistance. An intermediate approach which might prove to be very useful is to construct substrate plasmids containing sequences from Epstein-Barr virus, which have been shown to be located in the cell nucleus, chromatin-associated, and replicated with the chromosomes, thus being very good models for chromosomal DNA. These plasmids can be scored for recombination by isolation of cell DNA and transformation of *E. coli*.

It is also possible to optimize resolvase-Zif268 hybrid recombinases as exemplified for activity in mammalian cells, using methods analogous to those described above for the intact resolvase protein. The structure of Zif268 bound to DNA has been solved, (Elrod-Erickson, et al., 1996) and it is the focus of studies aiming to create engineered zinc finger proteins that can recognize any defined short DNA sequence. The properties of new variants of the hybrid recombinase will be studied first in *E. coli*, and *in vitro*, to allow for more detailed analysis and troubleshooting; suitable candidates will then be tested in mammalian cells. Further improvements in activity should be achievable by the application of mutagenesis followed by selection methods as described above. If these prototype recombinases are functional, it should be feasible to create analogous systems which recombine at a wide variety of synthetic sites, by replacing the natural Zif268 domain with known mutant versions that recognize different sequences (Chou & Isalan, et al., 2000 and

Wolfe et al., 1999). This would create the potential for applications of site-specific recombination technology where two or more recombination events can be promoted independently in the same cell.

A straightforward extension of this approach is to attempt to target site-specific recombination to natural sequences in genomic DNA. This would be achieved by replacing the Zif268 domain of the hybrid recombinase with an altered version engineered to recognize part of the target sequence. Serine recombinases are much more promising for this type of application than the tyrosine recombinases such as Cre; tyrosine recombinases are not obviously divisible into 'catalytic' and 'DNA recognition' domains, and require more homology between the recombining sites (6 - 8 bp, versus only 2 bp for serine recombinases). One application would be the targeting of a recombinase to a sequence in the human immunodeficiency virus (HIV) provirus. Excisive recombination between the two LTRs of the provirus (roughly 9 kbp apart) would eliminate it from the genome, thereby providing a potential basis for therapy. Additionally it may be possible to target other genomic sequences. One such application would be targeted integration of gene cassettes at bovine casein gene loci, with the aim of creating transgenic animals which can produce large quantities of pharmaceutically useful proteins (Wilmut et al., 1991).

Suitable sites for targeting will have a sequence resembling as far as possible the central basepairs of *res* site I, which are contacted by the N-terminal domain of resolvase and thus affect the efficiency of catalysis of strand exchange, flanked by sequences that can be recognized by one or two engineered versions of the Zif268 DNA-binding domain. Most potential target sequences will have insufficient dyad symmetry for strong binding by both subunits of a dimer of a single hybrid recombinase. However, evidence from current studies indicates that strong binding by

only one subunit of the resolvase dimer can lead to efficient recombination at a minimal site. A more sophisticated solution of this problem, if it turns out to be necessary, would be to express two versions of a hybrid recombinase, which could form heterodimers with appropriate sequence recognition properties. The Zif268 domain(s) of the hybrid recombinase may therefore be modified for optimal binding at one or both of these sequences, based on the latest published information (see Choo & Isalan, 2000). Sequence recognition could be improved if required, by established selection methods for zinc finger proteins (Isalan et al., 2001). Substrates containing two copies of the potential recombination site may be constructed and analysed as described above. In the case of targeted integration, efficiency will be improved by optimization of the sequence of the recombination site associated with the gene cassette to be integrated. It may also be possible to reduce or eliminate the possibility of reversal of the integration reaction, by design of the cassette-associated site, or by incorporating features from the ϕ C31 integration-specific serine recombinase system, which is also being actively studied for potential uses in mammalian cells (Groth et al, 2000).

Table 1

Template	Test plasmid	Method	Residues mutated	(Additional) mutations	Phenotype
wt	pGal(res x I)	oligo oligo	1-21 94-121	none D102Y D102Y A113T D102A M103I D102Y Q116H D102T M103T D102I M103W I97V G101S/ D102Y A113T	WWR WWR WWR WWR WWR WWR WWR
D102Y	pGal(I x I)	PCR-QG oligo PCR-QG	10-140 1-21 114-140	D102Y none R121K E124Q A117V I138V	WWR WWW WWP WWW
E124Q	pGal(res x I)	PCR oligo	1-185 94-121	(being sequenced!) I77T G70C L66N M76V F92S R121S I3F L66I M103I etc. D102Y G101S Q105L D102V M103W/ R119H	 WWP WWP WPR WWR??
		PCR-QG	10-140	G101S D102V Q105L S98G Q105L (?) E82G D102V/ M103I V107L (*)	WWP WWR WPR WPP WWP
		PCR-var	10-140	D102V	WWR
		PCR-dP	10-92	F92S	WPR
D102Y/ E124Q	pStr(I x I)	PCR-var?	1-185	A89T G101S V107M	WWW WWW WWW

* = + gd sequence 112-142

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Figure 1.

(D102)

(56)

Q19

Q14

(12)

Q14

Q14

Tax

10 0 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350 360 370 380 390 400 410 420 430 440 450 460 470 480 490 500 510 520 530 540 550 560 570 580 590 600 610 620 630 640 650 660 670 680 690 700 710 720 730 740 750 760 770 780 790 800 810 820 830 840 850 860 870 880 890 900 910 920 930 940 950 960 970 980 990 1000

shaded areas =
conserved residues

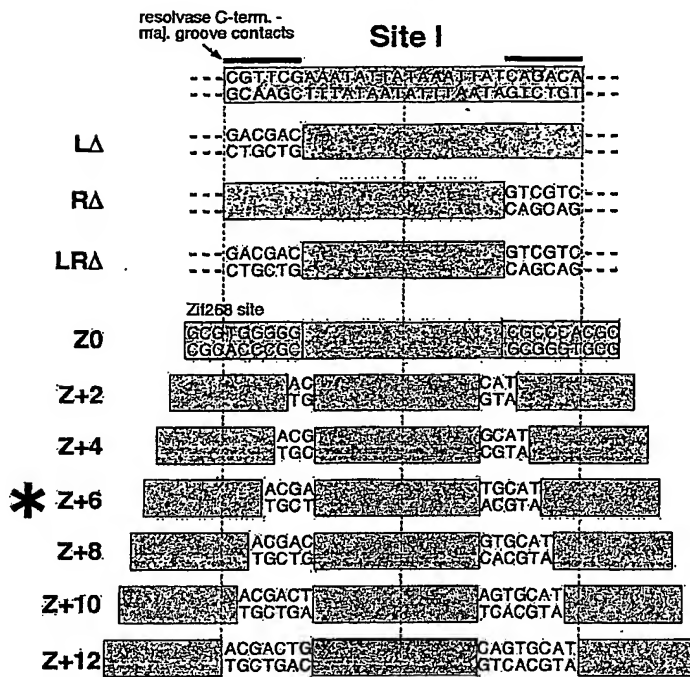
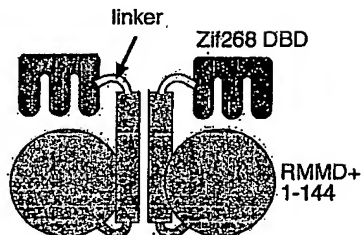
Alignment created by
Martin Barcock.

Underlined (red pos)
= highly conserved residues
(same).

(There are many more, some recombined, if we have

10 0 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350 360 370 380 390 400 410 420 430 440 450 460 470 480 490 500 510 520 530 540 550 560 570 580 590 600 610 620 630 640 650 660 670 680 690 700 710 720 730 740 750 760 770 780 790 800 810 820 830 840 850 860 870 880 890 900 910 920 930 940 950 960 970 980 990 1000

10 0 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350 360 370 380 390 400 410 420 430 440 450 460 470 480 490 500 510 520 530 540 550 560 570 580 590 600 610 620 630 640 650 660 670 680 690 700 710 720 730 740 750 760 770 780 790 800 810 820 830 840 850 860 870 880 890 900 910 920 930 940 950 960 970 980 990 1000

FIGURE 2a**FIGURE 2b****Z-resolvase****Linker sequences:**

TVDRSSDPTSQ (confection)

GSGGSG

GSGGSGGSG

* GSGGSGGSGGSG

GGGSGGG

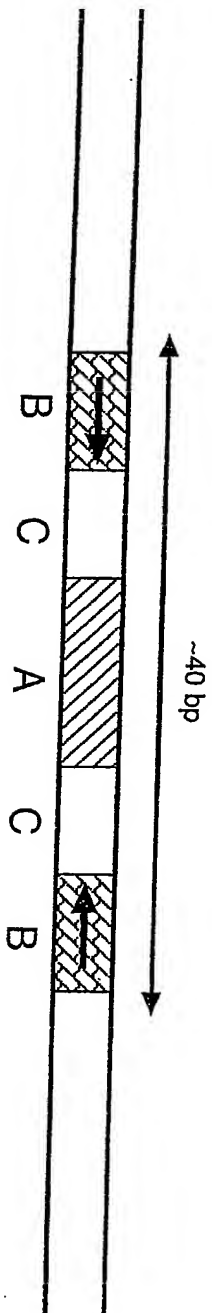
GGGSGGGGSGGG

NRVAQQLAGKQS (a la LacI)

SDYTQNNIHP (a la T4 Endo VII)

Figure 3

A generic hybrid recombination site



- A. The central sequence - interacts with catalytic domain and linker. Protein sequence may need to be optimized for any chosen sequence.
- B. Sequence motifs recognized by the DNA-binding domain. If they are the same, they must be in inverted repeat, as shown. For Zif268, the sequence is 9 bp long.
- C. Spacer sequence. No known sequence requirements at present, but length is important to optimize positions of B motifs.

FIGURE 4

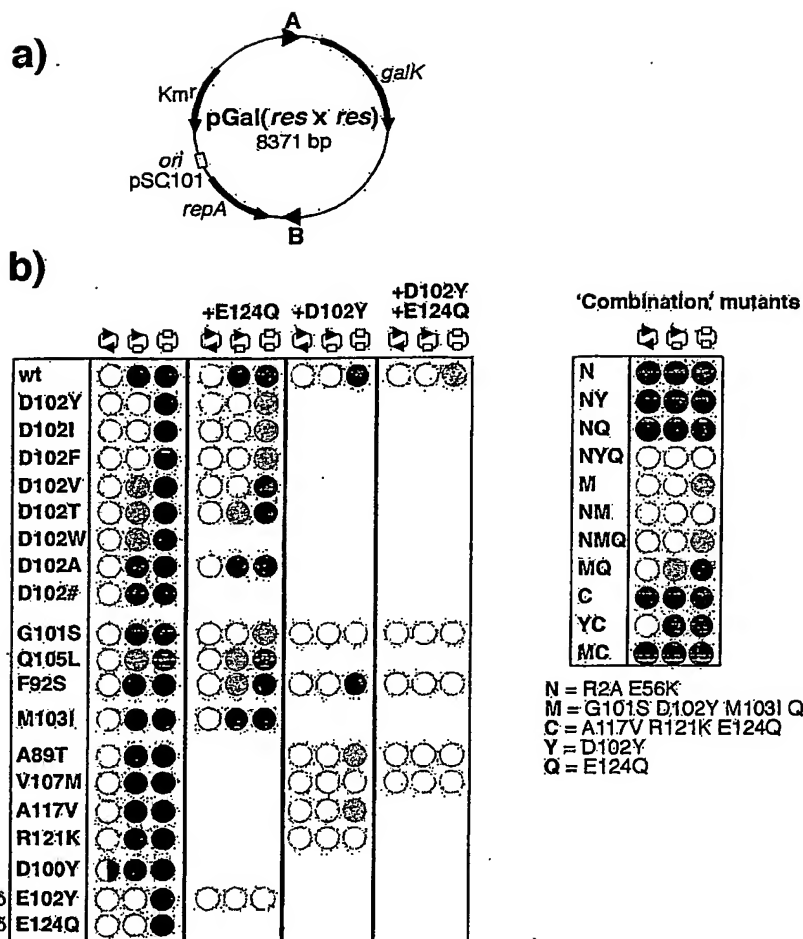


FIGURE 5

	<i>in vitro</i>			2x1 complex
wt.				
N	-	-	-	
NYQ	++++	++++	++++	
M	++++	+++	++	
NM	++	+++	++++	++++
NMQ	++	+++	++++	++++
MQ	++	+++	++++	
C	++++	-	-	
YC	++++	N.D.	+	
MC	++++	N.D.	+	

FIGURE 6a

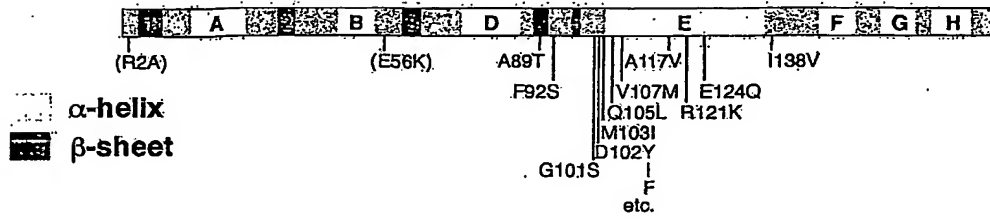
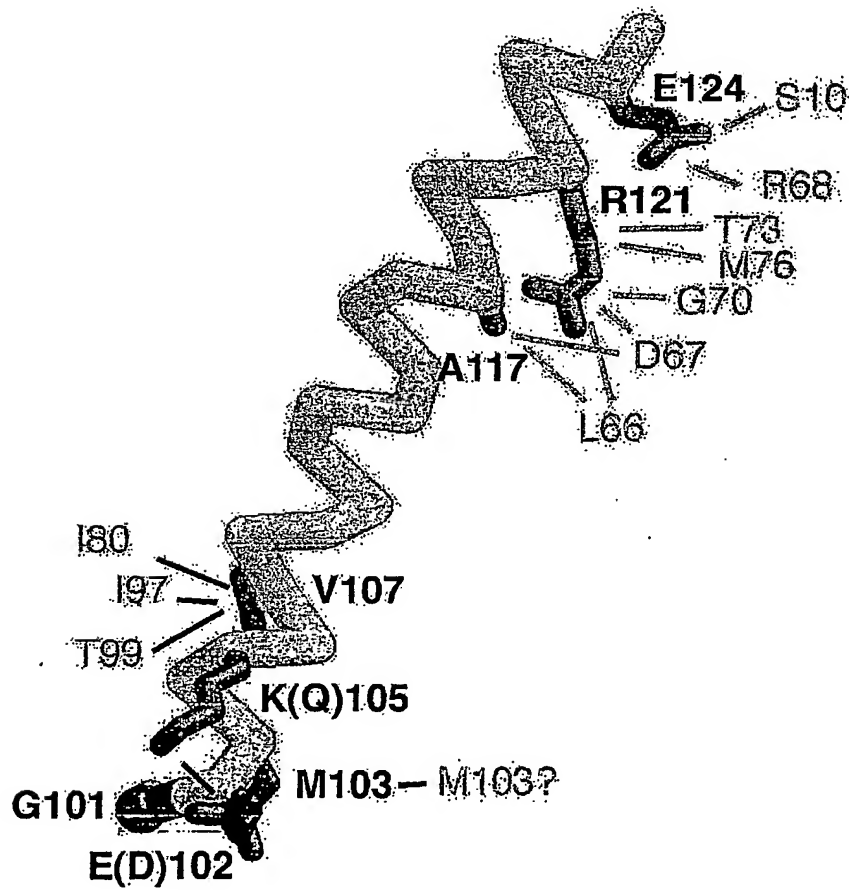


FIGURE 6b



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 03/04169

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/00 C12N15/09 C12N15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>GRAVEL ANNIE ET AL: "Point mutations in the integron intergrase IntI1 that affect recombination and/or substrate recognition"</p> <p>JOURNAL OF BACTERIOLOGY, vol. 180, no. 20, October 1998 (1998-10), pages 5437-5442, XP002289803</p> <p>ISSN: 0021-9193</p> <p>abstract</p> <p>-----</p> <p>-/--</p>	<p>1,2,</p> <p>7-17,46,</p> <p>47</p>

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search

8 October 2004

Date of mailing of the international search report

22 12. 2004

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INTERNATIONAL SEARCH REPORT

International Application No

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCHWIKARDI MICHA ET AL: "Site-specific recombination in mammalian cells catalyzed by gammadelta resolvase mutants: Implications for the topology of episomal DNA" FEBS LETTERS, vol. 471, no. 2-3, 14 April 2000 (2000-04-14), pages 147-150, XP002289804 ISSN: 0014-5793 abstract	1,2, 7-17,46, 47
A	----- ARNOLD PATRICIA H ET AL: "Mutants of Tn3 resolvase which do not require accessory binding sites for recombination activity" EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL, vol. 18, no. 5, March 1999 (1999-03), pages 1407-1414, XP002289805 ISSN: 0261-4189 the whole document	1,2, 7-17,46, 47, 52-62, 64,66, 67,69
X,P	----- AKOPIAN ARAM ET AL: "Chimeric recombinases with designed DNA sequence recognition." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA. 22 JUL 2003, vol. 100, no. 15, 22 July 2003 (2003-07-22), pages 8688-8691, XP002289806 ISSN: 0027-8424 the whole document	1,2, 7-17,46, 47, 52-62, 64,66, 67,69
A	----- BUCHHOLZ FRANK ET AL: "Alteration of Cre recombinase site specificity by substrate-linked protein evolution" NATURE BIOTECHNOLOGY, vol. 19, no. 11, November 2001 (2001-11), pages 1047-1052, XP002289807 ISSN: 1087-0156 abstract	1,2, 7-17,46, 47
A	----- EP 1 170 364 A (ARTEMIS PHARMACEUTICALS GMBH) 9 January 2002 (2002-01-09) abstract examples -----	1,2, 7-17,46, 47, 52-62, 64, 66-67,69

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 03/04169

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1,2, 7-17 (in part), 46,47,52-62 (in part),64 (in part),66-69 (in part)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1, 2, 7-17 (in part), 46, 47, 52-62 (in part), 64 (in part), 66-67 (in part), 68 and 69 (in part)

A serine recombinase comprising a catalytic domain and a DNA binding domain, wherein said catalytic domain is mutated at position G101; additional substitutions; hybrid recombinases containing the same mutated catalytic domain; a kit containing a DNA sequence encoding for said mutated domain.

2. claims: 3, 4, 7-17 (in part), 64 (in part), 48, 49, 52-62 (in part), 66-67 (in part), 68 and 69 (in part)

A serine recombinase comprising a catalytic domain and a DNA binding domain, wherein said catalytic domain is mutated at position Q105; additional substitutions; hybrid recombinases containing the same mutated catalytic domain; a kit containing a DNA sequence encoding for said mutated domain.

3. claims: 5, 6, 7-17 (in part), 50, 51, 52-62 (in part), 64 (in part), 66-67 (in part), 68 and 69 (in part)

A serine recombinase comprising a catalytic domain and a DNA binding domain, wherein said catalytic domain is mutated at position D102; additional substitutions; hybrid recombinases containing the same mutated catalytic domain; a kit containing a DNA sequence encoding for said mutated domain.

4. claims: 18-45, 68 and 69 (in part).

A hybrid recombinase comprising a catalytic domain from a serine recombinase connected by way of a linker to a heterologous DNA binding domain, nucleic acids encoding the same, host cells and/or vectors and kits containing the same.

5. claims: 63-66

A method for identifying a hyperactive mutant serine recombinase by mutating a serine and comparing its activity with the wild type recombinase.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 03/04169

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 1170364	A	09-01-2002	EP 1170364 A1	09-01-2002
			AU 8195901 A	21-01-2002
			CA 2415350 A1	17-01-2002
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			JP 2004502455 T	29-01-2004
			US 2004029107 A1	12-02-2004
			ZA 200300159 A	26-01-2004

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